**Full Paper:** The formation of microelectrostatic fields in biopolymers causes the adsorption of stain molecules in protein. The interactions between the dye alkali blue 6B (AB6B) and four proteins, bovine serum albumin (BSA), human γ-globulin (γ-G), horse myoglobin (Mb) and ovalbumin (OVA), were studied by the microphase adsorption/spectral correction technique. It was observed that the interactions obey the Langmuir isothermal adsorption. The Langmuir equilibrium constants of all the aggregates were determined. Interestingly, the adsorption of AB6B has no relation to the array sequence of amino acid residues, and an electrostatic field consists of constant amino acid residues. The novel method has been applied satisfactorily to the determination of the total protein content in two different samples.

Alkali blue 6B (C.I. 42750)
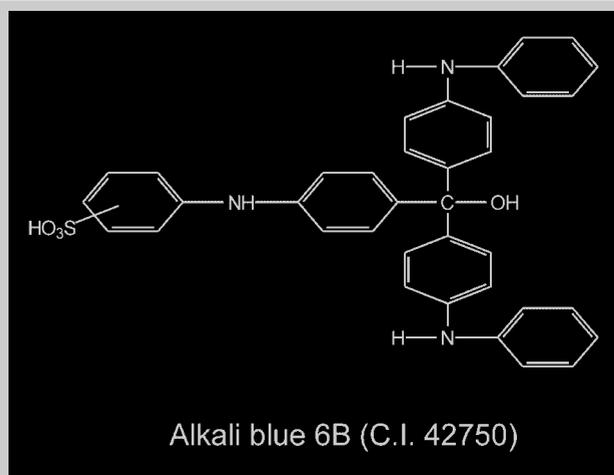
Langmuir Aggregation of Alkali Blue 6B in Proteins: Study and Application

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Introduction

The interaction of biopolymers with small ions and molecules has become increasingly important to chemists and physicians. Combinations of biopolymers and small anions and cations,[1] sulfonamides,[2] dyes,[3] alkyl sulfates,[4] fatty acids[5] and aromatic compounds[6] have already been described. Nowadays, more and more chemists and biochemists are interested in biopolymer (e.g., protein) chemistry and its study has gained more and more attention.[7] However, interactions between stain molecules and biopolymers have not been elucidated satisfactorily and earlier observations have not been explained clearly and reasonably, e.g., by using the Pesavento equation.[3b] The present study aims toward clarifying the general principles involved in protein/ stain interactions. For this purpose, several proteins, bovine serum albumin (BSA), human γ-globulin (γ-G), horse myoglobin (Mb) and ovalbumin (OVA), and the dye alkali blue 6B (AB6B) were selected. With the aid of simplifying assumptions, the number of AB6B molecules bound to each protein molecule and the adsorption constants of the aggregates were calculated from experimental measurements.

The quantitative determination is additionally useful[8] in clinical tests and for the accurate study of interactions with stain molecules.

A new method, the so-called microphase adsorption/ spectral correction technique (MPASC), is described and was applied to the interaction of AB6B (see below) with four different proteins. This method represents a very helpful experimental strategy in studying the absorption of chromophores or their metallic complexes in surfactant solutions.

Alkali blue 6B (C.I. 42750)
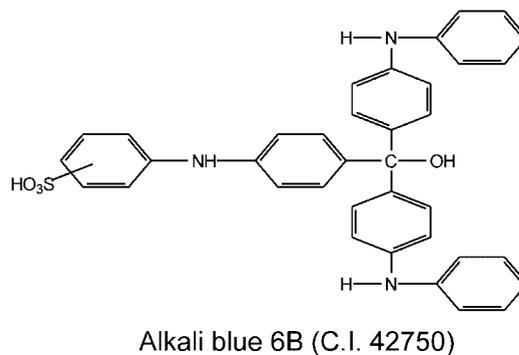
Experimental Part

Apparatus and Materials

Absorption spectra were recorded with a TU1901 spectro-photometer (PGeneral, Beijing) and independent absorbance was measured on a Model 722 Spectrophotometer (Shanghai 3rd Instruments). A DDS-11A conductivity meter (Tianjin Second Analytical Instruments) and a DJS-1 conductivity immersion electrode (electrode constant 0.98; Shanghai Tien-kuang Devices) were used to measure conductivities, in order to produce deionized water of $< 1 \ (\mu \cdot \Omega \cdot cm)^{-1}$. pH values were measured with a 320-S pH meter (Mettler-Toledo Instruments, Shanghai). The temperature was adjusted and kept constant with a Model 116R electrically heated thermo-static bath (Changjiang Test Instruments of Tongjiang, China).

Preparation of Solutions

Stock standard solutions of proteins were prepared by dissol-ving commercial BSA (Beijing Beitai Biochemical, Chinese Academy of Sciences), γ-G, (Serva, Heidelberg, Germany), Mb (Shanghai Chemical Reagents Center) and OVA (Shanghai Chemical Reagents Center) in deionized water. The protein content (w, $mg \cdot ml^{-1}$) of the solutions was determined and calculated according to the relation $w = 1.45 \cdot A_{280 \ nm} - 0.74 \cdot A_{260 \ nm}$[9] by measuring their absorbances ($A_{260 \ nm}$ and $A_{280 \ nm}$) at 260 and 280 nm by means of UV spectroscopy. The solutions were diluted to $0.100 \ mg \cdot ml^{-1}$ for use. The AB6B solution ($1.60 \ mmol \cdot l^{-1}$) was prepared by dissolving 0.3067 g of AB6B ($MW = 613.74$, dye content approx. 80%, Shanghai Third Reagents) in 100 ml of N,N'-dimethylformamide (DMF; Shanghai Central Chemical Factory) and then diluting to 250 ml with deionized water. Britton-Robinson buffer solu-tions between pH 1.8 and 7.24 were prepared to adjust the acidity of solutions. 2 mol/l NaCl was used to adjust the ionic strength of the aqueous solutions. Na_2-EDTA solution (1%) was prepared to mask the foreign metal ions possibly present in the samples. All reagents were used without further purification.

Measurements

Step 1: Preparation of Samples and Determination of Proteins

Two samples were analyzed. The first sample was prepared by diluting 1.00 ml of a children's drink to 100 ml with deionized water. The other sample was prepared with drinking water background to which the following compounds or ions were added: 1 mg each of ethanol, acetate, DANN and glucose, 0.5 mg each of Ca(II), PO_4^{3-} and Mg(II), 0.1 mg each of F^-, Fe(II), Mn(II) and Zn(II), and 0.05 mg each of Cu(II) and Pb(II). Drops of each of the four protein solutions were added and determined, respectively. In the analysis of the samples, 0.5 ml of the masking reagent, Na_2-EDTA solution (1%), were added to complex metal ions. The next successive operation accorded with (1) above.

Step 2: Aggregation of AB6B with Proteins

Given amounts of a solution of protein, 1.0 ml of Britton-Robinson buffer solution and appropriate amounts of the

AB6B solution were added to a 10-ml calibrated flask. The mixture was diluted to 10 ml with deionized water and mixed thoroughly. After 15 min, measurements were made against a blank solution, treated in the same way but without any protein.

Principal Equations and Calculation

In protein molecules, protonation of the amino groups ($-NH_3^+$) in polar amino acids results in the formation of a positive electrostatic film, whereas the deprotonated carboxylate groups ($-COO^-$) tend to form a negative electrostatic film. Proteins have complex spatial structures, e.g., winding, fold, coil and helix structures, and these cause crossings of the double electrostatic films to form numerous microelectrostatic fields. Ions can thus be attracted up to a kinetic equilibrium (Figure 1). The microelectrostatic fields are so narrow that dye molecules are adsorbed in a monolayer only. Therefore, aggregation is regarded to obey Langmuir isothermal adsorption.[10]

The adsorption equilibrium is expressed as: L (aqueous phase, C_L) $\Leftrightarrow$ ML_N (protein phase, C_M) in L/M solution. The Langmuir adsorption formula (Equation (1)) is used

$$\frac{1}{\gamma} = \frac{1}{N} + \frac{1}{KNC_L} \qquad (1)$$

where K is the equilibrium constant and C_L the concentration of excess L. N indicates the maximal adsorption ratio of L to M and γ the molar ratio of L adsorbed to M. K is calculated from Equation (1). Both C_L and γ are calculated by means of Equation (2)–(4)[11]

$$\gamma = \eta \times \frac{C_{L0}}{C_M} \qquad (2)$$

$$C_L = (1 - \eta)C_{L0} \qquad (3)$$

where

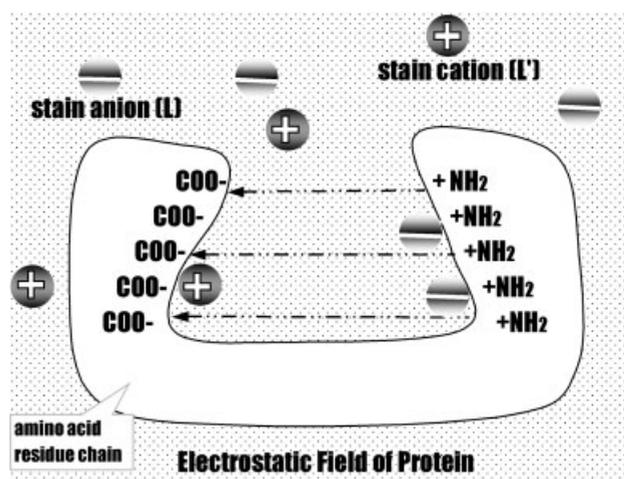$$\eta = \frac{A_c - \Delta A}{A_0} \qquad (4)$$

Figure 1. The formation of microelectrostatic fields in protein and the adsorption process of stain ions on microphase surface.

where C_M and C_{L0} are the concentrations of M and L added initially and η indicates the effective fraction of L. A_c, A_0 and ΔA are the real absorbance of the M/L product, the measured absorbance of the reagent blank against water and that of the M/L solution against reagent blank, directly measured at the peak wavelength λ_2. With increasing the concentration of L, γ will approach maximal N. A_c is calculated according to Equation (5)[11]

$$A_c = \frac{\Delta A - \beta \Delta A'}{1 - \alpha\beta} \qquad (5)$$

where $\Delta A'$ indicates the absorbance of the M/L solution measured at the respective valley absorption wavelength λ_1. Usually, α and β are correction constants[11] and are calculated by measuring directly ML_N and L solutions. In addition, the absorptivity (real $\varepsilon_r^{\lambda_2}$ not apparent $\varepsilon_a^{\lambda_2}$) of the adsorption product ML_N at λ_2 is also directly calculated by means of Equation (6)[12]

$$\varepsilon_r^{\lambda_2} = \frac{NA_C}{\delta\gamma C_M} \qquad (6)$$

where δ is the cell thickness (cm).

We found that the Pesavento hypothesis is identical with the Langmuir adsorption Equation (1). Consequently, the theoretical foundation of the Pesavento hypothesis derives from Langmuir isothermal adsorption.

Results and Discussion

Spectral Analysis and Determination of Correction Constants

The adsorption of AB6B to a protein (BSA as a representative) was investigated at pH 4.2. Absorption spectra of the BSA/AB6B solutions are shown in Figure 2. According to curves 1 and 2, maximal absorption for AB6B was observed at 570 nm and for the BSA/AB6B aggregate at 595 nm. The spectral red shift of the aggregate is only 25 nm. Therefore, the excess of AB6B will interfere with measuring the real absorbance of the aggregate. In curve 3, representing the relative absorption spectrum of the AB6B/protein solution against the AB6B solution without protein, the maximal absorbance value is located at 700.4 nm and the minimal one at 594.6 nm. Therefore, two wavelengths were used in this work. On the basis of curves 1 and 2 the correction coefficients were calculated to be $\beta = 0.289$ and $\alpha = 1.56$. Because of the high value for β, the spectral correction method was used instead of ordinary spectroscopy, and $A_c = 1.82(\Delta A - 0.289\Delta A')$.

Effect of pH on Aggregation of AB6B in Protein

The absorption of the BSA/AB6B solution was measured by varying the pH, and the real absorption of the aggregate was calculated. The results are shown in Figure 3. In curve 3, the real absorption of the aggregate approaches a maxi-
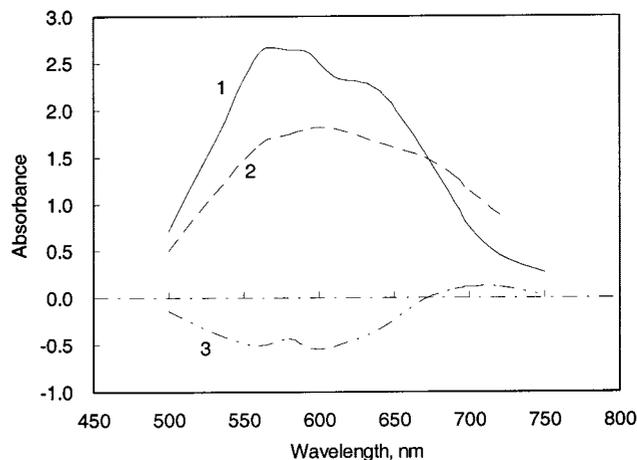

Figure 2. Absorption spectra of AB6B and its BSA solutions at pH 4.2: (1) AB6B solution (1.12 μmol), (2) solution, initially containing 1.12 μmol of AB6B and 1.5 mg of BSA, and (3) solution, initially containing 1.12 μmol of AB6B and 0.168 mg of BSA. Both (1) and (2) against water and (3) against the reagent blank.

mum between pH 3.8 and 4.56, which can be attributed to the easy formation of the AB6B anion. The AB6B anion is attracted closely to the microphase surface in protein molecules. In this work, pH 4.2 buffer solution was used.

Effect of Ionic Strength, Temperature and Interaction Time

The influence of the ionic strength of the solution on the real absorption of the AB6B/BSA solution is shown in Figure 4, curve 1. The adsorption ratio of AB6B to BSA increased with increasing the ionic strength to $< 0.4 \text{ mol} \cdot l^{-1}$ and then decreased rapidly above $0.4 \text{ mol} \cdot l^{-1}$. This can be attributed
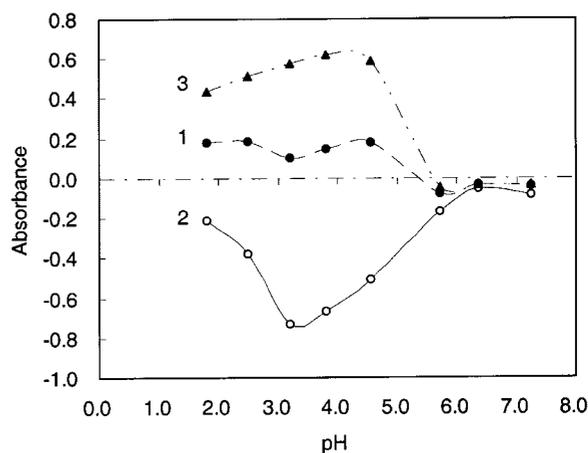

Figure 3. Effect of pH on absorption for the solution initially containing 1.12 μmol of AB6B and 0.168 mg of BSA at pH 4.2: (1) measured at 700.4 nm, (2) measured at 594.6 nm, and (3) real absorption of the aggregate at 700.4 nm. Both 1 and 2 against reagent blank.

Figure 5. (1) Effect of addition of 1.60×10^{-3} M AB6B on A_c of the BSA/AB6B aggregate at 700.4 nm, (2) γ^{-1} vs C_L^{-1} for the BSA (0.168 mg)/AB6B solution, (3) same as (2) but for the OVA (0.187 mg)/AB6B solution, (4) same as (2) but for the Mb (0.142 mg)/AB6B solution, and (5) same as (2) but for the γ-G (0.165 mg)/AB6B solution.

Effect of AB6B Concentration and Determination of Property Constants of the AB6B/Protein Aggregates

The absorption behavior of various protein solutions toward AB6B was measured by varying the added amounts of AB6B solution. This is shown for BSA/AB6B in Figure 5. The real absorbance approaches a maximum between 0.50 and 0.80 ml of 1.60×10^{-3} M AB6B (curve 1). In this study, 0.70 ml were added for the quantitative determination of protein, and γ and C_L values were calculated for each solution. Their relationship is also shown in Figure 5. All experimentally derived data points give linear graphs, indicating that the aggregation of AB6B in BSA, OVA, MB and γ-G
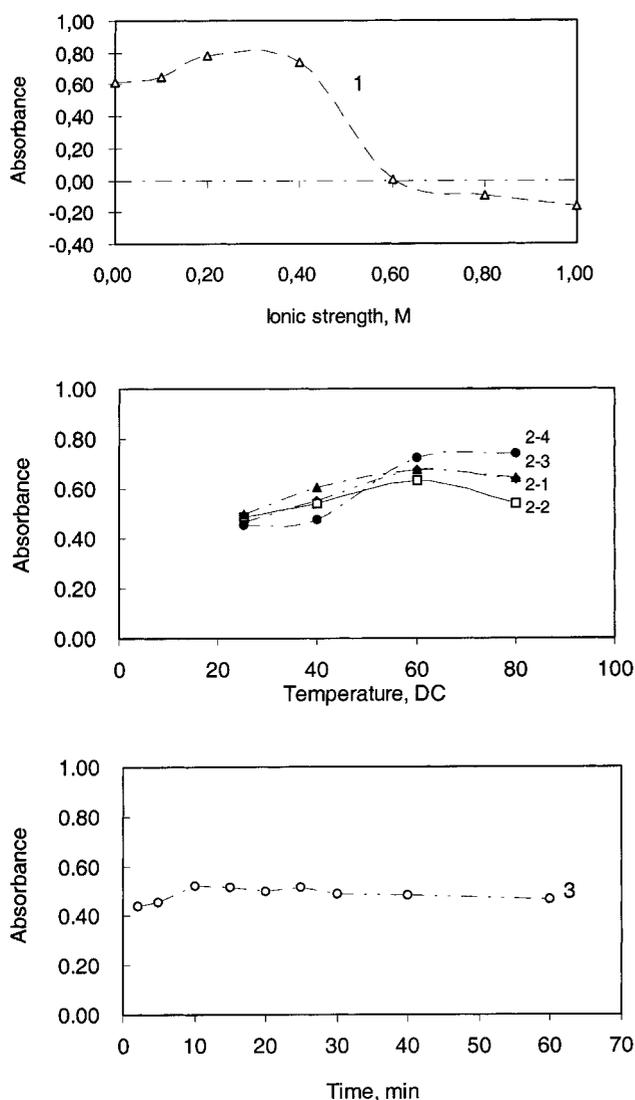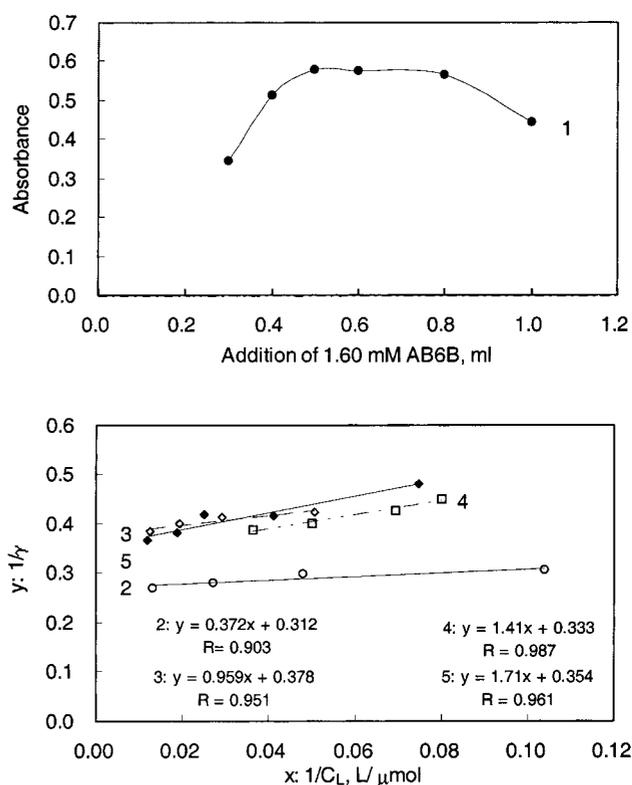
Figure 4. Effect of (1) ionic strength, (2-x) temperature (DC: degrees Centigrade), and (3) interaction time on the A_c of the aggregate at 700.4 nm where the solution initially contained: 1.12 µmol of AB6B and 0.168 mg of BSA (1, 2-1, 3), 1.12 µmol of AB6B and 0.150 mg of OVA (2-2), 1.12 µmol of AB6B and 0.280 mg of Mb (2-3), and 1.12 µmol of AB6B and 0.50 mg of γ-G (2-4).

to the self-aggregation of AB6B in the presence of NaCl. In highly concentrated NaCl solution, the aggregate becomes too big to be adsorbed in the electrostatic field of the protein.

The real absorption of the AB6B solution containing different proteins at various temperatures is shown in Figure 4, curves 2–1 to 2–4. All aggregates show higher absorbance at higher temperature. This indicates that AB6B is adsorbed tightly and that the temperature variations do not cause a desorption of AB6B, i.e., the aggregates are stable. Interaction between AB6B and BSA is complete within 10 min at room temperature, and remains almost constant for at least 30 min (Figure 4, curve 3).

Table 1. The linear regression equations for determination of proteins with AB6B at pH 4.2 in the presence of EDTA.

Protein	Linear scope mg/10 ml	Regression equation at 700.4 nm	Correlation coefficient
BSA	0–0.2	$A_c = 2.91 \times -0.008$	0.995
OVA	0–0.15	$A_c = 4.08 \times -0.013$	0.994
Mb	0.1–0.25	$A_c = 2.30 \times -0.204$	0.993
γ-G	0–0.12	$A_c = 3.41 \times -0.022$	0.992

Table 2. Determination of proteins in samples in the presence of EDTA (three determinations of each).

Sample no.	Added	Found
		mg
1	0.050 ml	0.103 ± 0.004; RSD. 3.9%
children's drink (protein content approx. 2%)	0.100 mg of OVA	0.114 ± 0.003; RSD 2.6%; rec. 111%
	0.100 mg of BSA	0.093 ± 0.003; RSD 3.2%; rec. 93.0%
2	0.100 mg of OVA	0.091 ± 0.004; RSD 4.4%; rec. 91.0%
drinking water background	0.200 mg of Mb	0.214 ± 0.01; RSD 4.7%; rec. 107%
	0.100 mg of γ-G	0.108 ± 0.004; RSD 3.7%; rec. 108%

obeys Langmuir monolayer adsorption. From the regression lines, the aggregation numbers of AB6B to BSA, OVA, Mb and γ-G, respectively, were calculated to be 3.2, 2.6, 3.0 and 2.8 µmol-AB6B/mg-protein, the adsorption ratios to be 218, 116, 81 and 424 (µmol AB6B)/(mg protein), and the adsorption constants of their aggregates to be $K = 1.99 \times 10^4$, 8.99×10^3, 8.76×10^3 and 1.38×10^3. The aggregation numbers (between 2.6 and 3.2) of AB6B per 1.0 mg protein are similar. As a consequence, it is suggested that the adsorption of AB6B has no relation to the array sequence of the amino acid residues of the protein, and that an electrostatic field consists of constant amino acid residues. According to Equation (6), the molar absorptivity of the aggregates was calculated to be $\varepsilon = 2.33 \times 10^6$, 1.65×10^6, 8.76×10^5 and 6.06×10^6 $1 \cdot mol^{-1} \cdot cm^{-1}$ at 700.4 nm, respectively. The spectral correction method proved advantageous for the determination of both adsorption ratio and adsorption constant as compared to classical methods based on molar ratios,[13] continuous variations,[14] etc.

Application to the Determination of Protein

Calibration Graph and Precision

The standard series of proteins was prepared and measured at pH 4.2, and 0.70 ml of 1.60 mmol · l⁻¹ AB6B were added. The values taken of the linear regression lines are summarized in Table 1. The slopes are between 2.30 and 4.08 and the mean value is 3.17. Ten replicated determinations of the reagent blanks gave a standard deviation of SD = 0.007. The detection limit of proteins for 3 times the SD was thus calculated to be 10 µg of BSA, OVA and γ-G and 100 µg of Mb in 10 ml of solution. Six replicated determinations of 0.168 mg of BSA were carried out. The mean value was 0.163 ± 0.006 mg.

Effect of Foreign Ions

By adding EDTA-Na₂ (0.5 ml of a 1% solution) to the protein solution, the influence of foreign substances on the determination of the protein was tested at pH 4.2. As EDTA forms complexes with most metal ions, high concentrations of metal ions may be allowed. The following amounts of compounds and ions did not affect the direct determination

of 0.168 mg of BSA (less than 10% error): 0.2 mg of Ca(II), Mg(II), F⁻, PO_4^{3-}, NH_4^+, $C_2O_4^{2-}$, Ac⁻, glucose, amino acid, 0.1 mg of ethanol, Mn(II), Fe(III), Al(III), Zn(II), 0.05 mg of Pb(II), Ni(II), Cd(II) and Cu(II). Cationic surfactant CTAB gave a negative error.

Determination of Proteins in Samples

Results of the sample determination (see Experimental Part) are given in Table 2. The protein content of the original children's drink was calculated to be 2.1%, which is close to the ''approx. 2% protein'' marked on the container. The recovery of OVA added to the first sample was 111%, the relative standard deviation (RSD) was 3.9%. The recovery of the various proteins added to the second sample was between 91.0 and 108%, with RSDs of less than 4.7%.

Conclusion

Investigations into the aggregation of AB6B in proteins and previous studies[15] support that the interaction between stain molecules and biopolymers can be described by means of Langmuir monolayer adsorption. Even though the MPASC technique does not show higher sensitivity than other methods such as RLS,[8b] it may meet the precision and accuracy criteria and offers additional benefits, such as simplicity and versatility. To our understanding the classical method may still play an important role in studying the interactions between biopolymers and small ions or molecules.

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